ALARM SUBSTANCES OF THE STINGLESS BEE,  
*Trigona silvestriana*

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Abstract—2-Nonanol, 2-heptanol, octyl decanoate, and octyl octanoate were identified from the heads of *Trigona silvestriana* workers. When presented at the nest, 2-nonanol, 2-heptanol, and the mixture of the four compounds elicited angular flights, landing, and buzzing of guard bees. Octyl octanoate elicited a weaker response. No response was given to octyl decanoate, to the ether solvent, or to the control volatile, vanillin.

Key Words—Alarm substances, nest defense, 2-heptanol, 2-nonanol, mandibular gland, Hymenoptera, Apidae, Meliponinae, stingless bees, *Trigona silvestriana*.

INTRODUCTION

*Trigona silvestriana* Vachal (Apidae: Meliponinae) readily mounts a mass biting attack against large mammals in the vicinity of its nest (Johnson, 1974). Here we report that attack is released by alarm substances found in worker heads, which house the large mandibular glands (Cruz, 1962; Michener, 1974) containing the alarm pheromones of stingless bees (Blum et al., 1970; Blum and Brand, 1972; Luby et al., 1973; Keeping et al., 1982).

METHODS AND MATERIALS

Collection of Bees for Chemical Analysis. In Guanacaste Prov., Costa Rica, individual worker bees were collected in a net and refrigerated. Heads of the
torpid bees were swiftly removed with a clean razor blade and sealed with a Microflame torch into glass ampoules containing dichloromethane while the bottom of the ampoule remained frozen by immersion in liquid nitrogen.

Sample Analysis. The sealed glass ampoules were cooled and opened. A sample of the solvent over the bee heads was analyzed by GC and GC-MS. In addition, heads were crushed in the storage solvent and that solution analyzed. There was some increase in peak height and an increase in components with retention times longer than 18 min (Varian 3700 programing conditions: 8-min hold at 50°C, 50–270°C at 10°C/min). Gas chromatographic analyses were conducted on either a Varian 3700 equipped with a 6-ft column with OV-17 as the stationary liquid phase or on a Hewlett-Packard 5830A equipped with a 20.3-
m-long, and 0.3-mm-diam capillary column coated with SE-52 and with a 20.0-
m-long, and 0.3-mm-diam capillary column treated with barium carbonate and coated with Silar 9CP. The programing conditions for the Hewlett-Packard 5830A were: 3-min hold at 50°C, 50–250°C at 3°C/min. GC-MS data were collected on a Hewlett-Packard 5985B GC-MS system, operated under standard Autotune conditions.

The earliest eluting peak (12.5% of the volatiles) was identified as 2-heptanol by comparison of GC retention times, coinjection of a known sample and the extract, and by comparison of the mass spectra. The next peak (12.7% of the volatiles) was identified in like manner as 2-nonanol. The next two peaks (23.9% and 10.0% of the volatiles) were suspected to be the octyl (large peak at 112 m/z) esters of octanoic acid (base peak at 145 m/z) and of decanoic acid (base peak at 173 m/z), respectively. The two esters were synthesized from the corresponding acid chloride and 1-octanol and were found to match in retention times, by coinjection and by comparison of the mass spectra. See Table 1 for retention times and mass spectral data.

To provide further confirmation of the identity of the two alcohols and the two esters, chemical ionization (methane) mass spectra were obtained. The alcohols characteristically gave a peak one mass unit less than the molecular weight, 115 for 2-heptanol and 143 for 2-nonanol. The base peak for 2-nonanol was 127, which corresponds to the ion produced by protonation of the alkene(s) formed by dehydration of the alcohol. Unfortunately, the mass range used for the set of experiments was 100–400 which missed the 99 peak for 2-heptanol. Octyl octanoate gave a base peak of 145 (octanoic acid + H+) and a protonated molecular ion at 257 (69%). Octyl decanoate gave a base peak of 173 (decanoic acid + H+) and a protonated molecular ion at 285 (58%).

No attempt was made to identify one component (23.4% of the volatiles) which eluted after the above two esters since the higher boiling compounds have not proven to be active as alarm pheromones. At the suggestion of a reviewer, chromatograms were obtained using a Silar 9CP column (McReynolds numbers 489, 725, 631, 913, 778) which differs considerably in retention behavior as
### Table 1. Retention Times and Mass Spectral Data

<table>
<thead>
<tr>
<th>Component</th>
<th>Retention time (min)</th>
<th>Base peak</th>
<th>Next three most abundant peaks and percentages</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Heptanol</td>
<td>3.7</td>
<td>45</td>
<td>69(37) 55(21) 43(17)</td>
</tr>
<tr>
<td>2-Heptanol(^c)</td>
<td>3.8</td>
<td>45</td>
<td>55(28) 83(22) 43(14)</td>
</tr>
<tr>
<td>2-Nonanol</td>
<td>9.8</td>
<td>45</td>
<td>69(57) 84(35) 55(31)</td>
</tr>
<tr>
<td>2-Nonanol(^c)</td>
<td>10.2</td>
<td>45</td>
<td>69(28) 55(18) 43(16)</td>
</tr>
<tr>
<td>Octyl octanoate</td>
<td>30.5</td>
<td>145</td>
<td>112(61) 83(46) 57(45)</td>
</tr>
<tr>
<td>Octyl octanoate(^c)</td>
<td>30.8</td>
<td>145</td>
<td>112(52) 57(52) 83(43)</td>
</tr>
<tr>
<td>Octyl decanoate</td>
<td>35.6</td>
<td>173</td>
<td>112(92) 83(65) 84(57)</td>
</tr>
<tr>
<td>Octyl decanoate(^c)</td>
<td>36.5(^d)</td>
<td>43</td>
<td>173(96) 51(81) 112(70)</td>
</tr>
</tbody>
</table>

\(^a\) As observed using the H-P 5830A system and the SE-52 column.
\(^b\) There is some variability in these data since spectra of knowns were not necessarily taken on the same day as the spectra of the components in the extract. The general pattern of the spectrum of a known and of the spectrum of a component were similar.
\(^c\) Known compound.
\(^d\) Matched with peak suspected to be octyl decanoate in a run for which the retention time of octyl octanoate was 31.2.

Compared to the SE-52 column (McReynolds numbers 32, 72, 65, 98, 67). The point of the experiments was to determine if some of the materials which eluted from the SE-52 column after the two esters would elute earlier from a column of vastly different polarity. The results were that only the previously identified compounds eluted early and in the same order. As before, the compounds were identified by coinjection of a known compound and the extract. The remainder of the volatiles were components with retention times longer than 40 min (HP-5830A programing conditions) or components of shorter retention times and individual percentages of 3% or less not detected using the HP GC-MS system but which were detected using the HP-5930A GC system.

The quantification of the components was accomplished by spiking each of 11 samples (one bee head per vial) with a known amount of 2-tridecanone. The retention time of 2-tridecanone falls between those of 2-nonanol and octyl octanoate. A total of 25 injections were done. Since there was considerable variation from bee to bee, the results are reported as averages and simple deviations from the averages. The values, per bee head, are: 2-heptanol, 4.7 ± 2.2 µg; 2-nonanol, 7.3 ± 2.8 µg; octyl octanoate, 6.4 ± 2.2 µg; octyl decanoate, 5.8 ± 1.5 µg.

**Preparation of Material for Bioassay.** 2-Heptanol and 2-nonanol were commercial samples which were used without further purification. The two synthesized esters, octyl octanoate and octyl decanoate, were shown to be greater than 99% pure by GC analysis on the capillary column and by GC-MS analysis. Solutions in ether for bioassay contained either 2-heptanol (0.95 g/liter), 2-non-
anol (0.93 g/liter), octyl octanoate (1.16 g/liter), and octyl decanoate (0.83 g/liter), or all four compounds in the above quantities. Also tested in the bioassay were ether alone and vanillin in ether (1.05 g/liter). Portions of the stock solutions were sealed in glass ampoules for transportation to the assay sites and opened only for immediate use in the bioassays.

**Bioassay.** The bioassay was performed January 9–12, 1983, at a nest of *Trigona silvestriana* 4 m high on a tree in Santa Rosa National Park, Guanacaste Prov., Costa Rica.

Caution was needed in administering the tests because the bees readily swarm in one’s hair and under the clothes, where they bite. The object of the tests was to investigate the bees’ response to the chemicals, not to the experimenter. The experimenter slowly approached the nest and took cover behind a tree 5 m in front of the nest entrance. No trial was run if there was the slightest sign of searching or alertness by guard bees. If the bees were calm, that is, the only activity was straightforward flight in and out of the nest tube, the test chemical was presented. Behind the tree the investigator pipetted 10 μl of test solution onto a 3.5-cm² piece of filter paper suspended by a short string from a long pole of roadside grass mounted on a stick. Within 5 sec the paper was smoothly thrust to a position 25 cm slightly upwind of the entrance, and held there for 1 min.

From the post 5 m in front of the nest tree, two kinds of alarm response could be reliably discerned. The big (7 mm) black workers of *T. silvestriana* that landed on the white paper could be readily counted. In addition, the presence or absence of short, erratic flights by bees in front of the entrance could be noted. These angular flight paths presented a sharp contrast to the smooth trajectories in and out of the nest tube normally made by the bees.

The following treatments were used, in order of presentation: (1) untreated paper, (2) ether, (3) vanillin in ether, (4) octyl octanoate in ether, (5) octyl decanoate in ether, (6) 2-nonanol in ether, (7) 2-heptanol in ether, and (8) a mixture of octyl octanoate, octyl decanoate, 2-nonanol and 2-heptanol in ether. The amounts tested were small, so as to be commensurate with the amount in one bee head. Vanillin was a control to test for the possibility that the bees would respond to the sudden presentation of any volatile chemical.

During the trials of 2-nonanol, 2-heptanol, or the mixture, bees sometimes located the experimenter. If this happened, data from that trial were discarded and another test attempt was made later in the day or on the following morning, after a shower and change of clothes. At least 15 min elapsed between trials or attempts; a clean paper, string, and pole were used each time. Over the four days, the series of chemicals was presented three times.

At the end of the second day a foraging *T. silvestriana* was located 10 m from the nest. Liquid expressed from its head was tested in the same way as the other chemical substances.
RESULTS

No response occurred in three trials to the untreated paper square, to the ether alone, or to the vanillin or octyl decanoate in ether. Bees landed on the paper or flew in angular, erratic paths when 2-nonanol, 2-heptanol, and the mixture were presented; the bees responded two out of three times when octyl octanoate was presented (Table 2). Landing was accompanied by buzzing, which could be felt along the pole when bees landed on the pole above the suspended paper.

A chi-square test of the landings allows us to reject the null hypothesis of no difference in the distribution of the 15 observed landings among the eight treatments ($\chi^2 = 26.068, 7 df, P < 0.005$). When treatments were combined into categories (controls, esters, and alcohol-containing treatments; or controls, esters, alcohols, and mixture), the differences remained significant at the $P < 0.005$ level. A Fisher exact probability test, furthermore, rejects the null hypothesis of no difference in proportion of trials with landing or erratic flight responses between the control and the experimental (bee chemical) trials ($P = 0.0005$).

The landing, flight, and buzzing responses were similar to those observed when the material from one fresh head was presented. During that 1-min test period, six landings occurred, buzzing was felt, and a large number of erratic flights were made.

Five of the nine trials of the alcohols or mixture were repeats of earlier

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of landings, trial</th>
<th>Total bees landing on paper$^a$</th>
<th>Erratic flights, trial</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ether</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vanillin</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Octyl decanoate</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Octyl octanoate</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2-Nonanol</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2-Heptanol</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Mixture</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

$^a$A chi-square test rejected the null hypothesis of no difference in the distribution of the 15 landings among the eight treatments ($\chi^2 = 26.068, 7 df, P < 0.005$).

$^b$: no erratic flights; +: 2 bees made erratic flights; ++: >2 bees made erratic flights (i.e., too many to count).
attempts during which the bees, after first responding to the test substance, found and attacked the experimenter behind the tree.

DISCUSSION

2-Heptanol and 2-nonanol are recognized alarm substances in bees of the family Apidae. Collins and Blum (1982, 1983) report that 2-heptanol and 2-nonanol from the sting extracts of honeybees release alarm behaviors in caged young workers. Keeping et al. (1982) found that 1-nonanol and 2-nonanol together made up 16% of the mandibular secretions of Trigona gribodoi; when worker heads were crushed near the nest, the bees closed the top of the entrance tube and ceased flight activity (Crewe and Fletcher, 1976). Luby et al. (1973) found significant amounts of 2-heptanol in the heads of Trigona mexicana and 2-heptanol and 2-nonanol in the heads of Trigona pectoralis. When these chemicals were wafted in front of the nest, the bees moved quickly, investigated, or attacked. 2-Heptanol and 2-nonanol also function as trail substances in Trigona spinipes, according to Blum (1979). Kerr et al. (1981) confirmed the trail function of 2-heptanol when, using an artificial trail of 2-heptanol drops, they led T. spinipes foragers to dishes of syrup the bees had visited a day earlier, but had abandoned.

Octyl octanoate and octyl decanoate are found in T. spinipes (Kerr et al., 1981) and are similar to the octyl hexanoate found in Trigona fulviventris (Johnson and Wiemer, 1982). The function of these esters is unknown. Octyl hexanoate did not appear to be an alarm substance in T. fulviventris, nor did it consistently act synergistically with the releaser alcohol, nerol. In T. silvestriana, octyl octanoate, but not octyl decanoate, elicited alarm behaviors, at least part of the time, but was not detectably synergistic with the alcohols. No function for these esters has been reported for T. spinipes. The esters may play some role in the marking of food sources by foraging bees; alternatively, the esters may represent material from the cuticle.

Overall, the similarity of the mandibular gland chemistry of T. silvestriana and T. spinipes is striking. The four compounds we found in T. silvestriana are also found in T. spinipes, plus 2-tridecanol, according to Kerr et al. (1981). These authors regarded the mandibular gland chemistry of T. spinipes as highly distinctive. This claim no longer holds, but they may be right in saying that T. spinipes lays a unique trail in Brazil, for T. spinipes is a South American species and T. silvestriana a Central American one.

Attraction, landing, buzzing, and angular flights are typical alarm behaviors in Trigona bees (cf. Blum et al., 1970; Luby et al., 1973; Johnson, 1980; Keeping et al., 1982; Johnson and Wiemer, 1982). Although mandibular movements could not be seen from a distance of 5 m, it is likely that T. silvestriana bees that landed on the test paper were also biting it. Biting is a prominent form
of nest defense in those *Trigona*, such as *T. silvestriana*, *T. corvina*, and *T. fuscipennis*, that have strong, sharp, 5-toothed mandibles and live in exposed or semiexposed nests (Johnson, 1974).

Of interest was the fact that the guard bees were more likely to locate the experimenter if 2-heptanol or 2-nonanol or the mixture had just been presented at the entrance. This suggests that the bees might be made more sensitive to provocative sights, smells, or vibrations (Free 1961) by prior exposure to alarm pheromone. Collins et al. (1980) found for honeybees a hierarchy of stages of arousal, elicited by different kinds of stimuli, from “alert” (which could be elicited by odors), through “activate” and “attract,” to “culminate,” in which bees actively defend the nest by biting, pulling hair, and stinging.

We propose that stingless bees also possess a hierarchy of stages of arousal in nest defense. A few guard bees, with the lowest thresholds, investigate a possibility. If they find additional stimuli, as from a moving, smelly, dark, hairy, warm animal (Free, 1961; Johnson, unpubl. data), they perform marking and other behaviors that alert other bees. These bees in turn may or may not be stimulated to arouse still others, depending upon the magnitude of the intruder problem. In this way the scale of the colony response is appropriately adjusted and maladaptive expenditure of energy is avoided. In the present study the number of bees around the filter paper was always small; only when additional stimuli from the experimenter were encountered was there sufficient provocation for an escalated attack response.

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**REFERENCES**


